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Overexpression of the HER2/*neu* proto-oncogene in breast cancer occurs in approximately 20% of cases and is related to poor patient prognosis. An improved understanding of how the HER2 proto-oncogene is regulated at the level of transcription would be beneficial for the rational design of therapeutic strategies aimed at reducing HER2 receptor expression. We have described and cloned a new Ets transcription factor, ESX, and shown that it binds to the Ets response element of the HER2 promoter (GAGGAA), where it causes upregulation of HER2 transcription. Elucidation of the three dimensional structure of ESX bound to the HER2 promoter is critical for development of novel therapeutics to block ESX transactivating function. We propose to determine crystallographic structures of defined protein domains of the multi-domain Ets transcription factor ESX alone and in complex with the HER2 promoter element. The ultimate goal of this research is to use structural analysis to define inter-domain interactions of ESX or specific contacts between ESX and the HER2/*neu* promoter, which can later be targeted for the design of small molecule modulators of ESX transactivating function. To this end we have expressed, purified and initiated crystal screens of full-length ESX and four peptides. Additional constructs of ESX will be tried, since to date no crystals have grown.

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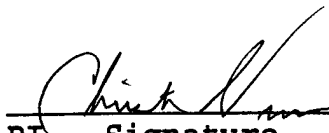

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SUMMARY OF RESULTS THROUGH JULY 1999

INTRODUCTION:

Overexpression of the HER2/*neu* proto-oncogene in breast cancer occurs in approximately 20% of cases and is related to poor patient prognosis. Although usually due to gene amplification of HER2, there is evidence for the contribution of enhanced gene transcription in the overexpression of this growth factor receptor. Therefore, an improved understanding of how the HER2 proto-oncogene is regulated at the level of transcription would be beneficial for the rational design of therapeutic strategies aimed at reducing HER2 receptor expression. Several Ets transcription factors are known to play a role in the malignant function of the HER2/*neu* oncogene. We have described and cloned a new Ets transcription factor, ESX, and shown that it binds to the Ets response element of the HER2 promoter (GAGGAA), where it causes upregulation of HER2 transcription. Elucidation of the three dimensional structure of ESX bound to the HER2 promoter is critical for development of novel therapeutics to block ESX transactivating function. We propose to determine crystallographic structures of defined protein domains of the multi-domain Ets transcription factor ESX alone and in complex with the HER2 promoter element. We will also attempt to determine the structure of the entire multi-domain protein with DNA. The ultimate goal of this research is to use structural analysis to define inter-domain interactions of ESX or specific contacts between ESX and the HER2/*neu* promoter, which can later be targeted for the design of small molecule modulators of ESX transactivating function. In our initial proposal, we planned to express, purify and begin crystallization trials of ESX during the first year. The following is a summary of our progress toward these goals.

BODY:

Technical Objective 1: Expression and purification of protein domains for structural analysis.

Months 0-12: Four fragments of ESX will be expressed and purified, as well as the full-length protein. Optimization of these procedures will be required to generate the necessary quantity of protein for crystallization trials.

In the initial proposal, four fragments of ESX were identified as potential candidates to screen for crystallization in addition to the full-length protein. These fragments were selected based on interesting homologies to other known protein domains, proteolytic data, and information obtained from genomic mapping. Full length ESX is 371 amino acids (aa) long and approximately 42 kD. The fragments selected for crystallization studies included:

a) DNA-binding domain (aa274-371)
b) DNA-binding domain + two-thirds SOX domain (aa206-371)
c) DNA-binding domain + SOX domain (aa188-371).
d) Pointed domain + one-third SOX domain (aa1-205).

Plasmids for expression of these fragments of ESX, in addition to the full-length protein, were constructed using pET vectors containing a T7 polymerase promoter and encoding a 6-histidine residue tag. Bacterial cells, BL21(DE3)pLysS, were induced with IPTG (isopropyl-ß-D-thiogalactopyranoside) to express T7 polymerase which drives high levels of RNA expression for the appropriate inserted fragment. Three to four hours following induction, the cells were harvested by centrifugation, resuspended in lysis buffer, and sonicated in order to lyse the cells. Following sonication the lysed cells and their cytosolic milieu were centrifuged to separate the soluble fraction from the insoluble fraction. In all cases the protein was resolved in the insoluble fraction, having been expressed in inclusion bodies, and hence required denaturing purification. The inclusion bodies were isolated using a sucrose separation step, in which the aggregates of protein settle through a layer of 52% sucrose upon centrifugation. Following this purification step, the protein was solubilized using 8M Urea. The denatured protein solution was purified using a Nickel column to which the N-terminal 6-histidine residue tag binds. Further purification by gel filtration was required to obtain >95% pure material suitable for crystallization trials. Following gel filtration, the protein required refolding, or removal of urea. This process of refolding has proven to be quite difficult. Several techniques have been tried including refolding while the protein was bound to the column, step dialysis in which the urea concentration is slowly reduced to 0, and simple dialysis. The resultant protein, in all cases, is marginally soluble and polydisperse, rendering unlikely the appearance of crystals. However, the protein is functional in DNA-binding assays. The low solubility of the protein is evidenced by its precipitation upon concentration. Dynamic Light Scattering experiments were performed on proteins following refolding and indicated that the solutions were not homogenous, and thus polydisperse. Soluble, monodisperse protein is still being sought. New constructs lacking the N-terminal 6-histidine tag have been made and their expression is currently being tested. Further experiments are necessary to determine whether the protein is soluble after expression, eliminating the need for denaturing purification, and hence, refolding. Eliminating the denaturing step will greatly increase the chances of obtaining crystals of these proteins.

Technical Objective 2: Crystallization of protein domains and protein-DNA complexes.

Months 0-12: As expression and purification are ongoing, crystal screens will be initiated to determine the fragment(s) suitable for advancement to Technical Objective 3. Optimization of the sparse matrix crystallization conditions for the appropriate fragments will likely be necessary.

Crystal screens have been initiated using full-length protein and two other fragments, (a) and (b) from above. Each of these fragments contains the DNA-binding domain, and has been tested alone, and in complex with a 16 base pair DNA substrate. The DNA substrate, a subset of the HER2 promoter element, was purified using HPLC. Both strands were purified separately, then annealed by placing in a 95°C water bath. The annealed oligomers were then desiccated, and resolubilized in water as needed. In initial screens of crystallization conditions, the hanging drop method was used. Protein or protein-DNA complex was mixed 1:1 with well buffer in 4 microliter drops. Drops were placed on siliconized cover slips and inverted over 1 ml of well buffer. Well buffers were obtained from commercially available sparse matrices containing greater than 10 distinct precipitants combined with different salts and additives (Hampton Research). To date there have been no visible crystals of the full length protein or either of the fragments alone or in complex with DNA. This result is unsurprising considering the data available from Dynamic Light Scattering experiments pertaining to the polydispersity of the proteins. New crystal trials will be initiated upon purification of protein that is soluble and monodisperse.

Technical Objective 3: Single crystal diffraction of protein domains and protein-DNA complexes.

Months 6-18: Selected crystals will be used to obtain diffraction patterns. Selection criteria will include crystal morphology and novel structural information to be gained.

As no crystals have been available to date, diffraction has not been possible. Once crystals are obtained, they will be subjected to diffraction on a laboratory based Raxis II or Raxis IV rotating anode x-ray generator equipped with a high speed area detector to screen for high quality single crystal diffraction data.

CONCLUSIONS:

Thus far, we are on track and meeting the schedule for the first 12 months provided in our initial proposal. While protein solubility and monodispersity have been issues thus far, the experience and knowledge gained from purification of the initial protein constructs will render current and future attempts much more expedient. The search for vectors, bacterial cells, or culture conditions (temperature, media-type, time of induction relative to the growth phase of the cells, etc.) that result in soluble, monodisperse protein will continue. There are many parameters that remain to be tested. Currently, three new plasmids have been constructed using a different vector than in previous trials. These plasmids have shown promising results in small-scale expression experiments that will hopefully persist during scaling-up of protein expression. In addition, the original DNA purification yielded a substantial amount of material that is ready for use in future trials.

ADDENDUM:

KEY RESEARCH ACCOMPLISHMENTS

- Expressed and purified full-length ESX and 4 protein fragments
  - Amino acids 274-371
  - Amino acids 206-371
  - Amino acids 188-371
  - Amino acids 1-205
- Purified a 16-base pair oligomer of DNA, a subset of the HER2 promoter element
- Screened crystallization conditions for full-length ESX and 2 protein fragments